BACTERIA AND CELLULAR ACTIVITIES. IV. ACTION OF TOXINS ON RESPIRATION AND HEMOLYSIS OF DOGFISH ERYTHROCYTES AND ON RESPIRATION OF MARINE EGGS [1, 2]

F. R. HUNTER, JANE A. BULLOCK, AND JUNE RAWLEY [3]

The Marine Biological Laboratory, Woods Hole, Mass., and the Department of Zoological Sciences, The University of Oklahoma

An attempt is being made in this laboratory to determine what effect bacterial toxins have on the functioning of cells. In the two preceding papers of this series (Hunter et al., 1949a, b) the action of seven toxins on the respiration and permeability of chicken erythrocytes was reported. In the present investigation the erythrocytes of the smooth dogfish, *Mustelus canis* and the unfertilized eggs of *Arbacia punctulata* and of *Asterias forbesei* were the cells studied.

Materials and Methods

The toxins were those previously used which were obtained locally and from the Lilly Laboratories and the Lederle Laboratories, to which the authors are indebted. Bacteriologically sterile techniques were used throughout except during the few minutes when hemolysis measurements were made. Tests for sterility were run at the end of each experiment as previously described (Hunter et al., 1949a). In addition, tests were also made for possible contamination by marine bacteria (see Waksman et al., 1933).

Oxygen consumption measurements were made using a Warburg apparatus at a temperature of 25° ± 0.1° C. Hemolysis times were measured using a photronic cell apparatus and a microammeter, since the more sensitive apparatus usually employed was not available. These measurements were made at room temperature which varied from day to day, but a water jacket surrounding the hemolysis chamber tended to minimize fluctuations during a series of readings. The time for hemolysis in 0.95 M ethylene glycol was measured in all cases.

Blood was procured under sterile conditions either by removal from the caudal vein with a hypodermic syringe, or by cutting the tail and allowing the blood to drain into a flask. Heparin was used as an anticoagulant in all experiments.

Immediately following the withdrawal of blood it was centrifuged at about 2000 r.p.m. for 10 minutes. The plasma and leucocytes were removed and the cells were carefully stirred. To 1 cc. of toxin was added 0.3 or 0.5 cc. of erythrocytes in each Warburg vessel which was immediately connected to its manometer and placed in the bath. Five to ten minutes were allowed for temperature equilibration, so that the initial readings were taken within 20 minutes or less of the time the toxins and cells were mixed. Controls were run using 1 cc. of sea water, 1 cc. of broth,

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[3] Present address—Department of Biology, Monmouth College, Monmouth, Ill.

or 1 cc. of formalized toxins in place of the 1 cc. of toxins. In some experiments readings were made continuously, while in others the manometers were opened for about an hour following the first hour's readings. Cell counts and hematrocrit determinations were made as previously described.

Hemolysis measurements were made as follows: Equal volumes of blood and toxins were mixed and allowed to stand at room temperature. Controls were run substituting sea water, formalized toxins, formalized broth and formalized sea water for the toxins. From time to time 0.1 cc. aliquots were removed and added to 10 cc. of 0.95 M ethylene glycol in the hemolysis chamber. The course of hemolysis was followed by reading the microammeter at five-second intervals. The total exposure time to the toxins varied, but the maximum was 48 hours.

Formalized "controls" were used in both the respiratory and hemolysis experiments. These were the same stock suspensions previously described (Hunter et al., 1949a). As was true in the preceding work (Hunter et al., 1949b), these attenuated toxins were more satisfactory as "controls" for the permeability studies than for the respiratory studies. However, these attenuated toxins were more satisfactory as "controls" in the present respiration studies. Apparently the oxygen consumption of dogfish erythrocytes is not inhibited by formalin to such an extent as the oxygen consumption of chicken erythrocytes.

Unfertilized eggs of *Arbacia punctulata* and of *Asterias forbesei* were obtained under bacteriologically sterile conditions, using a technique similar to that described by Tyler et al. (1938). The dry weight of the eggs was obtained by centrifuging 1 cc. samples of the egg suspensions using an air turbine, removing the supernatant fluid and drying to constant weight.

In the majority of experiments in which eggs were used, 1 cc. of the toxin was placed in each Warburg vessel, 2 cc. of an egg suspension were added, the vessels were attached immediately to the manometers and five minutes were allowed for temperature equilibration. The first reading was taken within 20 minutes of the time the first vessel was prepared. Readings were taken at 10 minute intervals over a period of several hours.

Results

Erythrocyte respiration

The results of a typical experiment in which dogfish erythrocytes were exposed to the toxins are summarized in Table I. Respiration was also measured in the presence of formalized toxins (26 days after mixing formalin and toxin). In the case of the three toxins which accelerate respiration (*M. aureus, Cl. tetani* and *C. diphtheriae*), cells in the presence of the corresponding formalized toxins consumed oxygen at essentially the same rate as sea water controls. The other formalized toxins inhibited the rate of respiration but none more markedly than formalized sea water.

Hemolysis times

The effects of the toxins on hemolysis of dogfish erythrocytes in 0.95 M ethylene glycol are shown in Table II. It can be seen that the toxins of *Cl. perfringens* are most effective in altering the surface of the cells, for a half hour exposure is sufficient to bring about a marked increase in the rate of hemolysis. A 9 hour ex-

TABLE I

A typical experiment showing the rates of respiration of dogfish erythrocytes in the presence of various toxins

Toxin	μL O_2 per cc. of cells per hour		Remarks	
	1st hour	3–5 hours	1st hour	3–5 hours
Control	80	70	———	———
M. aureus	140	108	Marked acceleration	Marked acceleration—slightly less than 1st hour
Cl. tetani	160	85	Marked acceleration	Slight acceleration
C. diphtheriae	120	75	Marked acceleration	Little or no effect
Cl. septicum	80	70	No effect	No effect
Cl. perfringens	80	0	No effect	Complete inhibition (Possibly associated with hemolysis of the cells)
B. cereus	65	60	Slight inhibition	Slight inhibition
Str. pyogenes	50	<20	Moderate inhibition	Marked inhibition

posure to the toxins of *Str. pyogenes* or *B. cereus* or a 23 hour exposure to the toxins of *Cl. tetani* produce a less marked increase in the rate of hemolysis. The formalized tetanal toxins had the same effect as the toxins themselves, while the formalized streptococcal toxins, and to a lesser extent, the formalized cereus toxins, had an intermediate effect. These data might indicate either that the formalin had not completely inactivated the toxins responsible for the change in the surface of the cells, or that the changes in the cells are brought about by something other than the toxins. Although additional experiments would be required to demonstrate conclusively the explanation for these observations, there is little reason for believing that substances other than the toxins would be influencing the cells in this manner. The composition of the broth in this case is unknown. However, none of the broths studied in this laboratory has had any observable influence on the osmotic behavior

TABLE II

Hemolysis times for dogfish erythrocytes in 0.95M ethylene glycol following exposure to various toxins

Time of exposure in hours	Organism producing toxin	Time in seconds for approximately 75 per cent hemolysis			
		Toxin	Control		
			Sea water	Formalized sea water	Formalized toxin
½	*Cl. perfringens*	11	31	31	29
9	*Str. pyogenes*	20	35	35	24
9	*B. cereus*	21	35	37	29
23	*Cl. tetani*	24	36	38	25
20	*Cl. septicum*	40	40	—	40
20	*C. diphtheriae*	30	35	25	30
20	*M. aureus*	35	35	25	30

of dogfish or other erythrocytes. In the case of tetanal toxin, the data obtained from the oxygen consumption studies suggest that the formalin does inactivate the toxin responsible for the change in respiration. As a tentative suggestion, therefore, one might assume that at least two tetanal toxins are present—one which accelerates respiration and is inactivated by formalin, and a second which alters the surface of the cell but which is not inactivated by formalin. It is hoped that future investigations will test the validity of this assumption.

The toxins of *Cl. septicum, C. diphtheriae,* and *M. aureus* have no effect on hemolysis following exposures of 20 hours.

Egg respiration

Figures 1 and 2 present the results of typical experiments using Arbacia and Asterias eggs respectively.

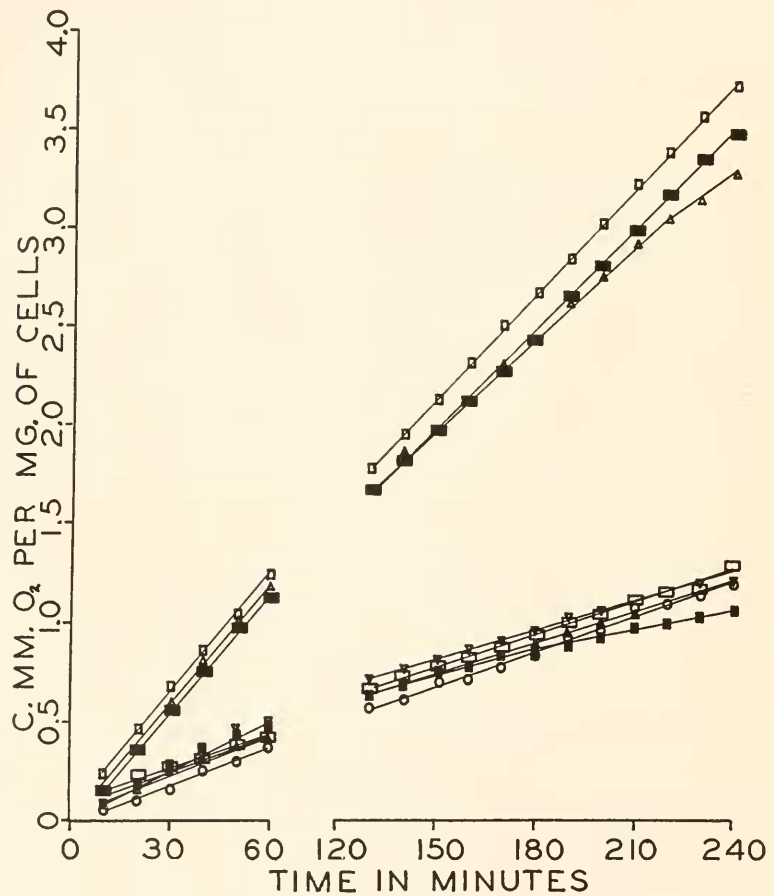

FIGURE 1. The effect of toxins on the oxygen consumption of unfertilized Arbacia eggs. Readings were taken for 60 minutes, then there was a 60 minute break and readings were again taken beginning at 120 minutes. □—*M. aureus;* ■—*Cl. tetani;* △—*C. diphtheriae;* ○—Sea water; ▽—*Cl. septicum;* ▲—*B. cereus;* ■—*Str. pyogenes;* ▭—*Cl. perfringens.*

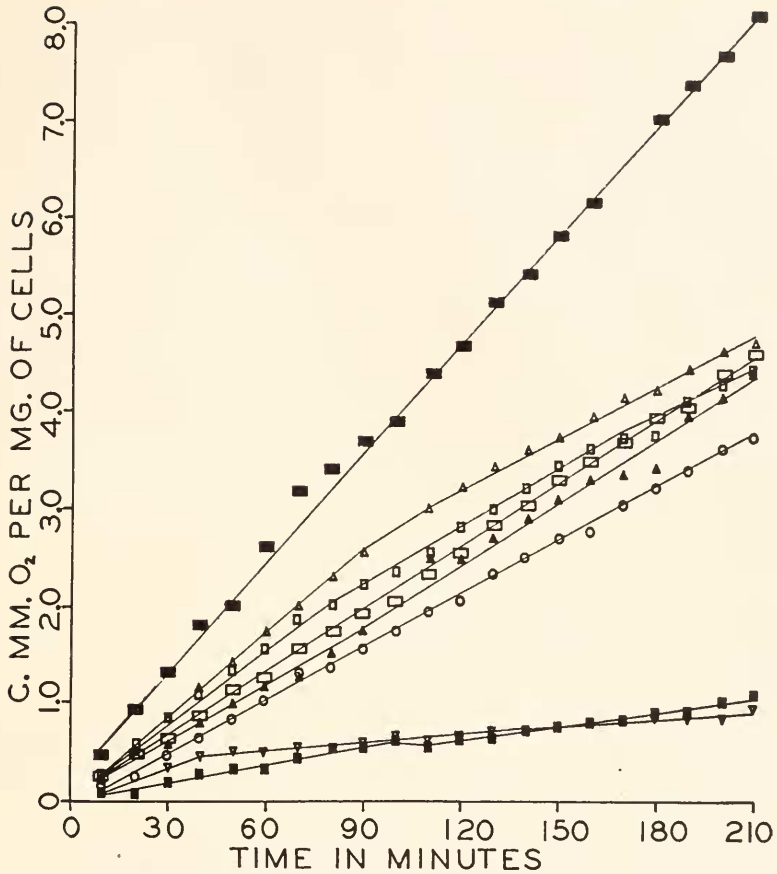

FIGURE 2. The effect of toxins on the oxygen consumption of unfertilized Asterias eggs. Readings were taken continuously for 210 minutes. ■—*Cl. tetani*; △—*C. diphtheriae*; □—*Cl. perfringens*; □—*M. aureus*; ▲—*B. cereus*; ○—Sea water; ■—*Str. pyogenes*; ▽—*Cl. septicum*.

These data are not conclusive since the heated toxins apparently had an effect similar to that of the toxins. However, it is interesting to note that the effects of these toxins on the eggs are in many respects the same as those on erythrocytes. In the case of Arbacia eggs, the toxins from *C. diphtheriae, Cl. tetani,* and *M. aureus* accelerate respiration. The significance of the lack of inhibition by the streptococcal toxins will be discussed in a future publication. In the case of Asterias eggs, the tetanal toxins clearly accelerate respiration; the diphtherial and staphylococcal toxins may accelerate slightly for the first hour or two; the perfringens toxins may accelerate slightly, while there is a suggestion of a delayed acceleration in the case of the toxins of *B. cereus*. The streptococcal toxins and those of *Cl. septicum* inhibit respiration.

DISCUSSION

A comparison between these data and those previously reported (Hunter et al., 1949a, b) shows that in general the action of the toxins on the four types of cells

studied is essentially the same. Marked acceleration of respiration was obtained only with micrococcal, tetanal and diphtherial toxins. The fact that in the presence of micrococcal toxins the respiration of chicken erythrocytes fell off, while this did not happen with the dogfish erythrocytes, may be explained by the hemolysis which occurred in the former case but not in the latter. In the presence of diphtherial toxins the initial acceleration is followed by a period during which there is no effect, or an inhibition of respiration of all but Arbacia eggs.

It is of interest to note that the rate of oxygen consumption of dogfish erythrocytes is considerably higher than that of chicken erythrocytes. Also, the sensitivity of the respiration of both types of cells to formalin suggests future experiments to study the respiratory mechanisms of these cells.

The relative resistance of the dogfish erythrocytes to the toxins containing lipid-splitting enzymes is worth noting. One of the most outstanding features of the action of toxins on the surface of chicken erythrocytes was the fact that the lecithinase in the toxins of *Cl. perfringens* and *B. cereus* and the lipase in the toxins of *M. aureus* markedly altered the chicken erythrocytes in a very short period of time. Much longer periods of exposure were required to alter the dogfish erythrocytes, particularly in the case of *M. aureus*.

Summary

1. The toxins obtained from *Micrococcus aureus, Clostridium tetani* and *Corynebacterium diphtheriae* accelerate the rate of oxygen consumption of dogfish erythrocytes initially.

2. The toxins obtained from *Streptococcus pyogenes* markedly inhibit the rate of oxygen consumption of these cells after approximately one hour's exposure.

3. The toxins obtained from *Clostridium perfringens, Bacillus cereus* and *Clostridium septicum* have little effect on the oxygen consumption of dogfish erythrocytes.

4. The time for hemolysis of dogfish erythrocytes placed in 0.95 M ethylene glycol is decreased by exposure to the toxins of *Streptococcus pyogenes, Clostridium perfringens* and *Bacillus cereus.*

5. There is a suggestion that the toxins of *Cl. tetani* have a similar effect, but formalized tetanal toxins also decrease hemolysis times.

6. The time for hemolysis of dogfish erythrocytes placed in 0.95 M ethylene glycol is not altered by the presence of the toxins of *Clostridium septicum, Corynebacterium diphtheriae* or *Micrococcus aureus.*

7. The toxins of *Clostridium tetani, Micrococcus aureus* and *Corynebacterium diphtheriae* increase the rate of oxygen consumption of both Arbacia and Asterias eggs.

8. The toxins of *Clostridium perfringens* increase the rate of oxygen consumption of Asterias eggs but have little effect on the respiration of Arbacia eggs.

9. The toxins of *Streptococcus pyogenes* decrease the rate of respiration of Asterias eggs but have little effect on Arbacia eggs.

10. The toxins of *Bacillus cereus* have little influence on the respiration of either Arbacia or Asterias eggs.

11. The toxins of *Clostridium septicum* inhibit the respiration of Asterias eggs but have little influence on Arbacia eggs.

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